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# SIV/HIV *nef* Recombinant Virus (SHIVnef) Produces Simian AIDS in Rhesus Macaques

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The simian immunodeficiency virus (SIV) *nef* gene is an important determinant of viral load and acquired immunodeficiency syndrome (AIDS) in macaques. A role(s) for the HIV-1 *nef* gene in infection and pathogenesis was investigated by constructing recombinant viruses in which the *nef* gene of the pathogenic molecular clone SIVmac239 *nef* was replaced with either HIV-1SF2*nef* or HIV-1SF33*nef.* These chimeras, designated SHIV-2nef and SHIV-33nef, expressed HIV-1 Nef protein and replicated efficiently in cultures of rhesus macaque lymphoid cells. In two SHIV-2nef-infected juvenile rhesus macaques and in one of two SHIV-33nef-infected juvenile macaques, virus loads remained at low levels in both peripheral blood and lymph nodes in acute and chronic phases of infection (for >83 weeks). In striking contrast, the second SHIV-33nef-infected macaque showed high virus loads during the chronic stage of infection (after 24 weeks). CD4+ T-cell numbers declined dramatically in this latter animal, which developed simian AIDS (SAIDS) at 47–53 weeks after inoculation; virus was recovered at necropsy at 53 weeks and designated SHIV-33Anef. Sequence analysis of the HIV-1SF33 *nef* gene in SHIV-33Anef revealed four consistent amino acid changes acquired during passage *in vivo.* Interestingly, one of these consensus mutations generated a tyr-x-x-leu (Y-X-X-L) motif in the HIV-1SF33 Nef protein. This motif is characteristic of certain endocytic targeting sequences and also resembles a *src*-homology region-2 (SH-2) motif found in many cellular signaling proteins. Four additional macaques infected with SHIV-33Anef contained high virus loads, and three of these animals progressed to fatal SAIDS. Several of the consensus amino acid changes in Nef, including Y-X-X-L motif, were retained in these recipient animals exhibiting high virus load and disease. In summary, these findings indicate that the SHIV-33Anef chimera is pathogenic in rhesus macaques and that this approach, i.e., construction of chimeric viruses, will be important for analyzing the function(s) of HIV-1 *nef* genes in immunodeficiency *in vivo,* testing antiviral therapies aimed at inhibiting AIDS, and investigating adaptation of this HIV-1 accessory gene to the macaque host. 

## INTRODUCTION

Highly manipulatable non-human primate models are critical for elucidating mechanisms of pathogenesis in AIDS and for development of antiviral therapies and vaccines. In macaques, human immunodeficiency virus type-1 (HIV-1) produces a low-level transient infection with no signs of immunodeficiency disease (Agy *et al.,* 1992). Simian immunodeficiency virus (SIV), a primate lentivirus genetically related to HIV, infects and causes a fatal AIDS-like disease in susceptible macaques (Gardner and Luciw, 1997). Thus SIV infection of macaques has become a useful and important animal model to analyze virus/host interactions, including the function(s) of viral genes and regulatory elements in viral transmission, cell-tropism, viral load and persistence, and pathogenesis, as well as evaluation of antiviral drugs and vaccines. Because HIV-1 and SIV have a similar genetic organization and display homology throughout their genomes (Shugars *et al.,* 1993), it is possible to construct recombinant (chimeric) viruses that are replication-competent *in vitro* in tissue culture cells by substituting a gene(s) of an infectious SIV clone with its HIV-1 counterpart (Shibata *et al.,* 1991). Such chimeras between SIV and HIV-1 are designated SHIV (reviewed in Overbaugh *et al.,* 1996). SHIV clones containing the HIV-1 *tat, rev, vpu,* and *env* genes readily infect several macaque species, and, after one or more passages through animals, exhibit a pathogenic phenotype (Joag *et al.,* 1996; Reimann *et al.,* 1996; Luciw *et al.,* 1999). Accordingly, SHIV clones have become important for analyzing viral phenotypes controlled by the HIV-1 *env* gene and for the evaluation of anti-HIV-1 vaccines in the macaque model of lentivirus infection and AIDS.

Both HIV-1 and SIV *nef* have been extensively analyzed *in vitro* in cell culture systems and *in vivo* in macaques to define the function(s) of this viral gene. *In vitro* functions ascribed to the *nef* gene are down-regulation of cell surface CD4 antigen, degradation of MHC-I proteins, modulation of cell activation pathways, and enhancement of virion infectivity (reviewed in Saksela, 1997; Cullen, 1998). *Nef* is not required for viral replication in T-cell lines, although this gene enhances replication in unstimulated primary lymphocyte and macrophage cultures (Miller *et al.,* 1995; Alexander *et al.,* 1997).

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Adult macaques infected with a genetically engineered SIV mutant, containing a deletion in the *nef* gene (SIVmac239Δnef), showed low virus load and no disease (Kestler *et al.,* 1991). Additionally, point mutations in SIVmac239 *nef* that affect certain Nef functions reverted *in vivo* in juvenile macaques, and these reversions were linked to increases in viremia and development of fatal immunodeficiency (Sawai *et al.,* 1996; Khan *et al.,* 1998). Interestingly, some human long-term nonprogressors harbor HIV-1 with deletions in *nef* (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995). Although these aforementioned studies supported the importance of *nef* in viral pathogenesis, fatal disease was produced in newborn macaques infected with high doses of SIV clones containing a deletion in the *nef* gene (Baba *et al.,* 1995; Wyand *et al.,* 1997). Nonetheless in adults, *nef* is viewed as a significant facilitator of viral disease (Baba *et al.,* 1999).

Comparison of *nef* of SIVmac239 and several HIV-1 isolates reveals similarities and differences. The Nef proteins of both viruses contain a myristylated N terminus and show homology, largely in the central domain (Shugars *et al.,* 1993). Nef of SIVmac contains ~50 additional amino acids near the N terminus that are not found in HIV-1 Nef. Recent studies, aimed at defining functional domains of both SIVmac239 and HIV-1 Nef, suggest that not all such domains are homologous (Greenberg *et al.,* 1998; B. M. Peterlin, personal communication). The interchangeability of SIV and HIV-1 *nef* has been investigated by constructing recombinant viruses. Two *in vitro* studies demonstrated that HIV-1 *nef,* when substituted for SIV *nef* in the SIVmac239 clone, enabled the chimeric virus to replicate efficiently in cultures of macaque primary lymphocytes and a macaque T-cell line (Alexander *et al.,* 1997; Sinclair *et al.,* 1997). Macaques experimentally inoculated with SHIV clones containing HIV-1 *nef, tat, rev,* and *env* genes revealed that these animals showed low virus loads with no clinical signs of immunodeficiency disease (Igarashi *et al.,* 1994; Shibata *et al.,* 1997). Animals in these studies were followed for only 4–6 months; thus, the long-term potential for viral persistence and pathogenesis was not addressed. Because the chimeric viruses tested in animals in the aforementioned studies contained several HIV-1 genes, it is not feasible to assess the contribution(s) of HIV-1 *nef* alone to the virus/host relationship. Accordingly, we constructed SHIV clones that replaced *nef* of SIVmac239 with *nef* from each of two distinct HIV isolates, HIV-1$_{SF}$2 from an individual in the early stages of AIDS (Levy *et al.,* 1984) and HIV-1$_{SF}$33 from a patient in the terminal stage of AIDS (Cheng-Mayer, 1991). These chimeras, designated SHIV-2nef and SHIV-33nef, were analyzed for Nef function *in vitro* and tested *in vivo* in juvenile rhesus macaques. One animal infected with SHIV-33nef showed high virus load and developed simian AIDS (SAIDS). Virus recovered at necropsy from this animal contained four to seven consistent amino acid changes in Nef; this recovered virus subsequently produced high viral loads and SAIDS in additional juvenile macaques. The results of this study indicate that chimeric viruses are a means to analyze changes in HIV-1 *nef* sequences that might be important for adaptation to a heterologous host, and to explore HIV-1 *nef* function *in vivo.*

## RESULTS

### *In vitro* characterization of SHIV-2nef and SHIV-33nef recombinants

Infectious virus was rescued from the SHIV-2nef and SHIV-33nef clones by transfection of cultures of CEMx174 lymphoid cells (see Fig. 9). Extensive cytopathology developed in cultures 3–4 days after transfection with each chimeric virus, as well as with SIVmac239nef+ and SIVmac239Δnef. Cell-free stocks of all these viruses were prepared in CEMx174 cultures, and infectivity titers were measured by end-point dilution in microtiter wells containing CEMx174 cells. To assess the ability of the SHIVnef recombinants to replicate in macaque PBMC, cultures of stimulated lymphocytes were inoculated with cell-free stocks of SHIV-2nef and SHIV-33nef; comparisons were made to PBMC cultures infected with SIVmac239nef+ and SIVmac239Δnef. Levels of viral replication were measured, with an ELISA for SIV p27 gag antigen, in culture supernatants for 17 days. The two chimeric viruses replicated in macaque PBMC to levels similar to SIVmac239nef+ and SIVmac239Δnef (Fig. 1). Thus, the hybrid structures of SHIV-2nef and SHIV-33nef allowed for efficient replication *in vitro* in primary lymphoid cells.

The function of the *nef* gene in the SHIVnef chimeras was analyzed in an *in vitro* kinase assay, which assessed the ability of Nef to associate with and activate a cellular serine/threonine kinase, designated Nef-associated kinase (NAK) and p21-activated kinase (PAK) (Sawai *et al.,* 1996). CEMx174 cells were infected with SHIV-2nef and SHIV-33nef, cell lysates were prepared at 5–7 days after infection, and NAK and PAK activities were tested in coimmunoprecipitates prepared with anti-HIV-1 Nef antibody. Both SHIV-2nef- and SHIV-33nef-infected cells showed similar levels of NAK and PAK activity (Fig. 2A). In additional experiments, the levels of Nef protein, determined by immunoblot analysis of infected cell lysates, was similar for both chimeric viruses (these authors, data not shown).

### Analysis of SHIVnef clones in macaques

To explore the ability of the chimeric viruses to replicate *in vivo* and to test their pathogenic potential, two juvenile rhesus macaques were inoculated intravenously with stocks of SHIV-2nef, and two additional animals were later infected with SHIV-33nef. These experiments included comparisons with macaques infected with

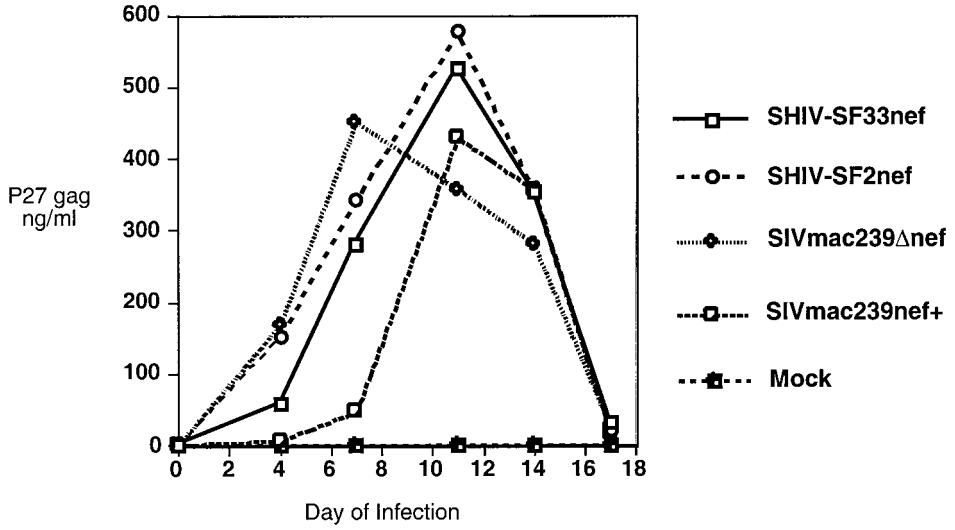


FIG. 1. Replication of SHIVnef chimeras *in vitro* in macaque PBMC. SHIV-2nef and SHIV-33nef were compared to SIVmac239nef+ and SIVmac239Δnef in *in vitro* cultures of rhesus PBMC. Each culture, containing $10^6$ PBMC in 1 ml medium, was inoculated at a m.o.i. of 0.2 TCID50 of each virus per cell. The level of viral replication was determined by measuring SIV $p27^{gag}$ antigen levels in culture supernatants.

SIVmac239nef+, which causes SAIDS, and the virulence-attenuated clone SIVmac239Δnef. All animals were monitored for (i) signs of clinical disease, i.e. fever, lymphadenopathy, splenomegaly, weight loss, hematologic abnormalities, and CD4+ T-cell decline; (ii) cell-associated virus in PBMC and lymph nodes and cell-free virus in plasma; (iii) viral RNA expression and cellular targets in necropsy tissues by *in situ* hybridization; and (iv) anti-viral antibody responses to virion core protein (p27 gag) by ELISA.

*SHIV-2nef.* Two macaques (Mmu 26120 and Mmu 26167) infected with SHIV-2nef showed no clinical or laboratory signs of immunodeficiency or AIDS-like disease. The CD4/CD8 ratios, CD4+ and CD8+ T-cell numbers remained within reference range from before inoculation (Week 0) values through the 83 weeks post-inoculation (PI) (data not shown). Both cell-associated and plasma virus loads in the two SHIV-2nef-infected animals were very low throughout the course of infection (Fig. 3A). Lymph node biopsies from Mmu 26120 and Mmu 26167 showed only a mild follicular hyperplasia at 2 and 8 weeks PI (data not shown), when viral replication was low in PBMC and lymph node cells (Fig. 3A, Table 1). At 83 weeks PI, these SHIV-2nef-infected animals were euthanized; post-mortem examinations for both animals were unremarkable.

*SHIV-33nef.* Two juvenile macaques (Mmu 27621 and Mmu 27747) were inoculated with the second chimera, SHIV-33nef. Mmu 26721 remained healthy for >105 weeks PI, with no discernible clinical or pathologic abnormalities. Virus levels, both cell-associated and in plasma, in this animal were low in the acute and chronic stages of infection (Fig. 3A, Table 1). CD4+ and CD8+ T-cell counts remained within the reference range throughout the course of infection; and at week 8 and 32 PI, peripheral lymph nodes from Mmu 27621 exhibited only mild follicular hyperplasia (data not shown).

In contrast, the second SHIV-33nef-infected animal, Mmu 27747, developed diarrhea, marked CD4+ T-cell decline, neutrophilia, and peritonitis between 47 and 53 weeks PI and was euthanized at 53 weeks PI (see below). At 2 weeks PI, this macaque exhibited relatively high virus load in peripheral blood, lymph nodes, and plasma (Fig. 3A, Table 1). Subsequent to the 2-week time point, virus load declined and then exhibited a sustained increase at 24 weeks PI and onward. This pattern of viremia, high in the acute stage, with a decline followed by an increase to high levels, was similar to the pattern observed in Mmu 27098, a positive control animal infected with the pathogenic clone SIVmac239nef+ (Fig. 3A, Table 1). Mmu 27747 showed mild follicular hyperplasia in peripheral lymph nodes at 8 weeks PI (data not shown). However, striking changes were observed in lymph node architecture at 32 weeks PI; marked lymphofollicular hyperplasia was characterized by expanded follicles with thin mantle zones, lymphoblastic lymphoid cells in expanded germinal centers, and multiple follicles present deep in the medulla (data not shown). These hyperplastic lesions in lymph nodes of Mmu 27747 correlated with high virus load (Fig. 3A, Table 1).

At the time of euthanasia at 53 weeks PI, Mmu 27747 displayed a moderate lymphopenia (870 cells/$\mu$l), neutrophilia (12,470 neutrophils/$\mu$l), and severe decrease in CD4+ T cells (56 CD4+ T cells/$\mu$l), in association with other signs of SAIDS, including diarrhea. Mmu 27747 also had a persistent neutropenia at weeks 12–42 weeks PI. All other hematologic parameters were within reference ranges. Pathologic findings at necropsy included septicemia, bacterial peritonitis, bacterial tonsillitis, lymphocytic gastroenteritis,

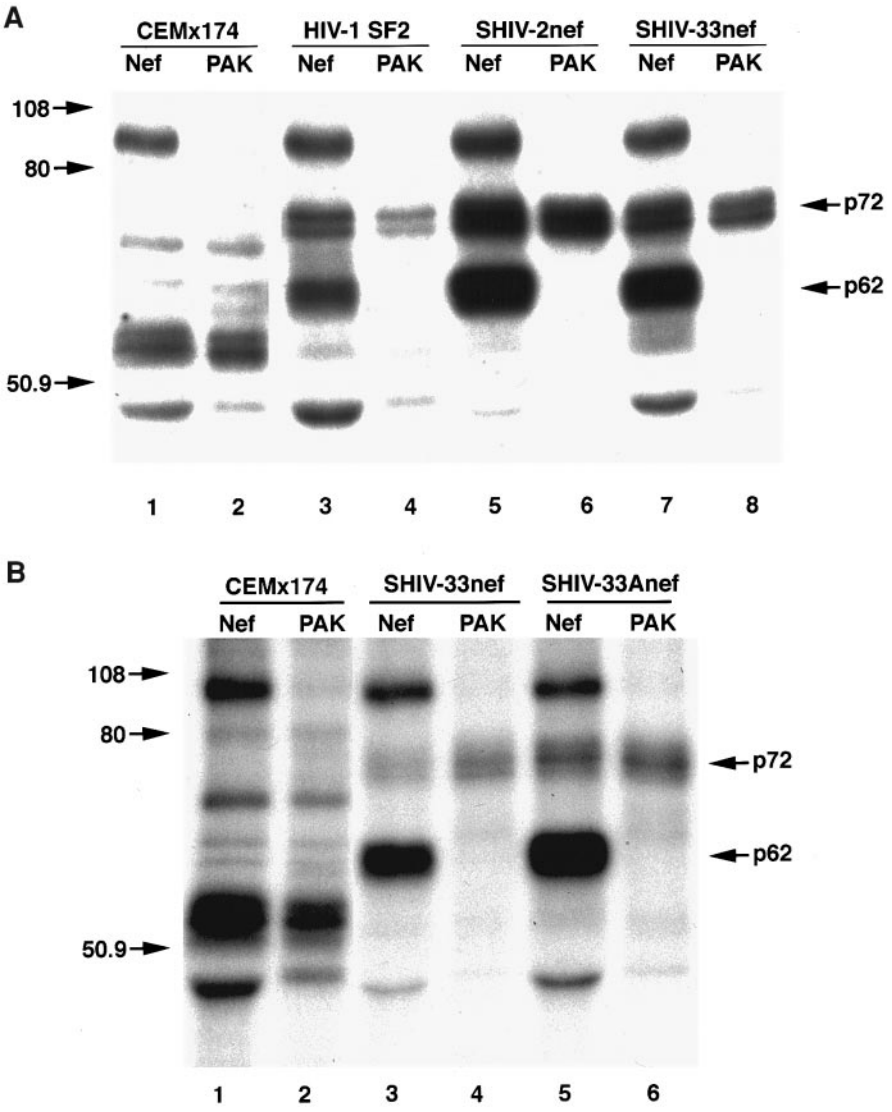


FIG. 2. Assay for Nef-associated kinase (NAK) and p21-activated kinase (PAK) activation in SHIVnef-infected cells. Lymphoid cell lines were infected with various viruses, and levels of NAK and PAK were determined in Nef and PAK immunoprecipitates by *in vitro* kinase assays (Sawai *et al.,* 1996). The phosphorylated cellular proteins p62 (indicator of NAK activity) and p72 (indicator of PAK activity) are labeled. (A) Lanes 1 and 2, uninfected CEMx174 cells. Lanes 3 and 4, HUT78 cells infected with HIV-1SF2. Lanes 5 and 6, CEMx174 cells infected with SHIV-2nef. Lanes 7 and 8, CEMx174 cells infected with SHIV-33nef. (B) Lanes 1 and 2, uninfected CEMx174 cells. Lanes 3 and 4, CEMx174 cells infected with SHIV-33nef. Lanes 5 and 6, CEMx174 cells infected with SHIV-33Anef.

marked lymphoid hyperplasia in the majority of lymph nodes, lymphoid depletion in selected gastrointestinal lymph nodes, and chronic cholangiohepatitis. Tissues taken at necropsy from Mmu 27747 were also examined for viral RNA expression by *in situ* hybridization. There was evidence of viral replication in multiple organs. Large numbers of SIV RNA-positive cells were detected in peripheral, abdominal, and thoracic lymph nodes, lung, and spleen, and within an intrabdominal abscess. Combined *in situ* hybridization and immunohistochemistry revealed that the majority of infected cells were macrophages with fewer numbers of CD3+ T cells positive for viral RNA (Fig. 4). Disease in susceptible primates infected with pathogenic lentiviruses is characterized by CD4 T-cell depletion in lymphoid tissues and persistent infection of macrophages (Embretson *et al.,* 1993; Veazey *et al.,* 1998). Virus recovered from PBMC obtained at necropsy from Mmu 27747 was designated SHIV-33Anef.

Mmu 26939, infected with SIVmac239Δnef, showed an increase of virus load at 72 and 105 weeks PI; this animal was euthanized at 105 weeks PI, and found to display widespread lymphoid hyperplasia. Sequence changes in virus from a necropsy sample of Mmu 26939 revealed several changes that produced a truncated form of SIV Nef (Sawai *et al.,* 1999).

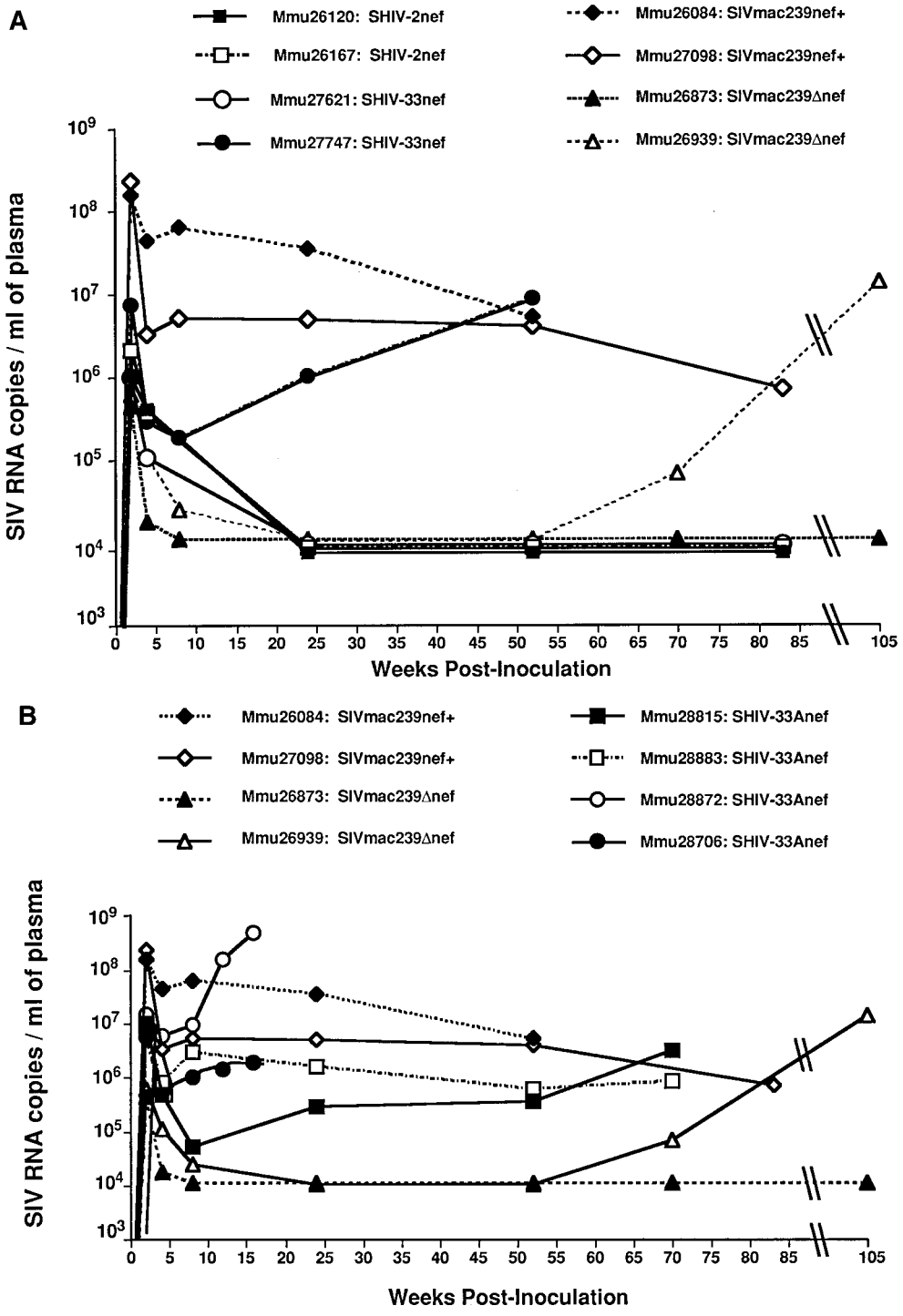


FIG. 3. Plasma virus load in SHIV-nef-infected macaques. SIV RNA in plasma of macaques infected with SHIVnef chimeras were compared to macaques infected with pathogenic wild-type virus SIVmac239nef+ and nef-deleted virus, SIVmac239Δnef. Plasma virus was assessed by measuring SIV RNA levels using bDNA signal amplification assay; the lower limit of sensitivity is 10,000 RNA copies/ml. (A) Macaques infected with the clones SHIV-2nef and SHIV-33nef. (B) Macaques infected with SHIV-33Anef (i.e., virus recovered from necropsy of Mmu 27747, the animal with SAIDS).

## Pathogenesis in macaques infected with SHIV-33Anef

To test the pathogenic potential of SHIV-33Anef, four juvenile rhesus macaques (Mmu 28815, Mmu 28883, Mmu 28706, and Mmu 28872) were inoculated with SHIV-33Anef, which was the uncloned virus recovered at necropsy from Mmu 27747, the animal that died of SAIDS at 53 weeks PI. In these four recipients of SHIV-33Anef, levels of viral RNA in plasma (by bDNA assay) and levels of cell-associated virus in PBMC and lymph nodes were markedly higher than in animals infected with

TABLE 1

Viral Load in Peripheral Blood Mononuclear Cells (PBMC), Lymph Node Cells (LNMC) in SIV & SHIV Infected Macaques

| | Weeks post-inoculation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | 24 | 32 | 42 | 52 | 83 |
| SHIV2nef | | | | | | | | | |
| Macaque 26120 | | | | | | | | | |
| PMBC | 100 | 320 | 2 | <1 | <1[a] | <1 | <1[a] | <1 | <1 |
| LNMC | 462 | ND | 100 | 316 | ND | <1 | ND | <1 | <1 |
| Macaque 26167 | | | | | | | | | |
| PBMC | 320 | 32 | 3 | <1 | <1[a] | 3 | 3 | <1 | <1 |
| LNMC | 464 | ND | 32 | 3 | ND | 10 | ND | <1 | <1 |
| SHIV33nef | | | | | | | | | |
| Macaque 27621 | | | | | | | | | |
| PBMC | 316 | 47 | 275 | <1[a] | 5 | 3 | 42 | 3 | |
| LNMC | 316 | ND | 32 | ND | ND | 1,000 | ND | 100 | |
| Macaque 27747 | | | | | | | | | |
| PBMC | 10,000 | 170 | 2,138 | 215 | 2,754 | 19,953 | 2,291 | 3,162* | |
| LNMC | 21,54 | ND | 5,000 | ND | 21,380 | >1,000,000 | ND | 46,420* | |
| SHIV33Anef | | | | | | | | | |
| Macaque 28815 | | | | | | | | | |
| PBMC | 4,217 | 1,000 | 15 | 2,154 | 46 | 32 | 316 | 464 | 1,000[d] |
| LNMC | 31,623 | 464 | ND | 42 | ND | 46 | ND | 1,000 | 2,154[d] |
| Macaque 28883 | | | | | | | | | |
| PBMC | 10,000 | 2,154 | 3,162 | 7 | 3,162 | 585 | 2,540 | 422 | 1,708 |
| LNMC | 215,443 | 464 | ND | 10,000 | ND | 3,162 | ND | 1,708 | ND |
| Macaque 28706 | | | | | | | | | |
| PBMC | 3,162 | 457 | 2,154 | 3,162[c] | 2,154 | | | | |
| LNMC | 3,162 | 464 | ND | | | | | | |
| Macaque 28872 | | | | | | | | | |
| PBMC | 10,000 | 3,162 | 1,000 | 10,000[c,*] | | | | | |
| LNMC | 21,544 | <10[b] | | | | | | | |
| SIVmac239nef+ | | | | | | | | | |
| Macaque 26084 | | | | | | | | | |
| PBMC | 47,000 | 32,000 | 2,200 | 15,000 | 10,000 | 700 | 31,600 | 1,400* | |
| LNMC | 4,700 | ND | 17,000 | 46,000 | ND | 5,900 | ND | 3,750* | |
| Macaque 27098 | | | | | | | | | |
| PBMC | 22,000 | 470 | 100,000 | 1,000 | 4,650 | 316 | 3,160 | 1,000 | 1,000* |
| LNMC | 4,700 | ND | 4,700 | 21,500 | ND | 215,000 | ND | 4,650 | 320* |
| SIVmac239Δnef | | | | | | | | | |
| Macaque 26873 | | | | | | | | | |
| PBMC | 3,162 | 100 | 100 | <1 | <1 | <1 | <1 | <1 | |
| LNMC | 215 | ND | 5 | ND | 2 | ND | ND | 2 | |
| Macaque 26939 | | | | | | | | | |
| PBMC | 146,780 | 3,162 | 68 | 46 | 7 | 3 | 2 | 22 | |
| LNMC | 14,678 | ND | 464 | ND | 215 | ND | ND | 1,708 | |

*Note.* Viral load expressed as $TCID_{50}$ per $10^6$ PBMC or $10^6$ LN cells. ND, not done; *, virus load at necropsy. Virus loads in plasma were below limits of detection for SHIVnefSF2 and SIVmac239Δnef.

[a] $10^7$ PBMC positive for virus.

[b] Inadequate sample.

[c] Week 12 samples.

[d] Week 76 samples.

SIVmac239Δnef (Fig. 3B and Table 1). In addition, virus load in these four SHIV-33Anef-infected macaques were of equal magnitude or greater than those in Mmu 27747, which received the original SHIV-33nef clone and developed SAIDS (Fig. 3A and Table 1).

Importantly, the clinical condition of the SHIV-33Anef-infected animals was monitored. At 12 and 60 weeks PI, respectively, Mmu 28872 and Mmu 28815 developed several clinical signs. Mmu 28815 showed weight loss, inappetence, persistent watery diarrhea, nasal discharge, severe neutropenia (369 neutrophils/$\mu$l), and decreasing CD4/CD8 T-cell ratios. Despite 10 weeks of supportive therapy, clinical signs in Mmu 28815 increased in severity culminating in depression, dehydration, anorexia, severe muscle wasting, cachexia, pale mucus membranes, decreased CD4/CD8 ratio of 0.5,

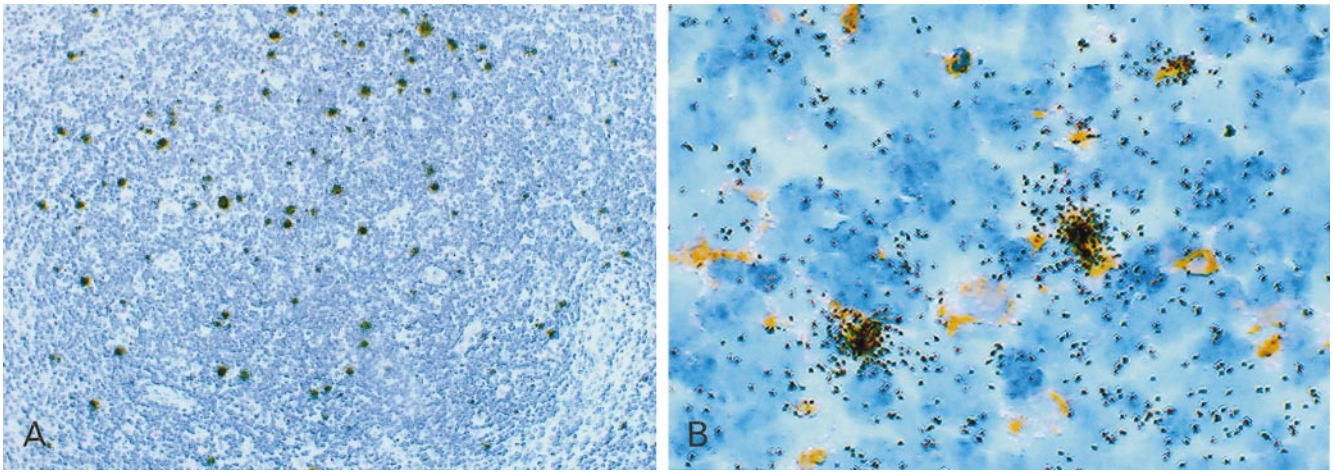


FIG. 4. Localization and expression of SHIVnef nucleic acid in lymph node taken at necropsy (53 weeks PI) from Mmu 27747, the SHIV-33Anef-infected macaque that developed SAIDS. (A) SHIVnef-infected cells in lymph node of Mmu 27747 by *in situ* hybridization. Note black grains indicate virally infected cells. (Hematoxylin counterstain, ×250 magnification). (A) Colocalization of SHIVnef nucleic acid in lymph node macrophages. Combined *in situ* hybridization for viral nucleic acid (black grains) and immunohistochemistry for macrophage marker HAM-56 (red–orange stain) shows virus positive macrophages in lymph node of Mmu 27747 (AEC chromogen with hematoxylin counterstain, ×400 magnification) (Veazey *et al.,* 1998).

CD4 T-cell lymphopenia (CD4 T-cells 423/$\mu$l), marked hypoproteinemia, and a left-shifted neutrophilic leukocytosis characteristic of an inflammatory response. This animal was euthanized at 79 weeks PI. Mmu 28872 showed a rapid deteriorating clinical course after infection with SHIV-33Anef. At 4 weeks PI, a transient anemia and hypoproteinemia was observed. At Weeks 12 and 14, Mmu 28872 exhibited decreased appetite, mild nonregenerative anemia, and hypoproteinemia in the absence of diarrhea. By 16 weeks PI, this animal was anorectic and cachectic due to marked weight loss; additional abnormalities included CD4 T-cell lymphopenia, mildly decreased CD4/CD8 ratio from baseline, moderate nonregenerative anemia (hemoglobin 8 gm/dl), severe hypoproteinemia, hypoalbuminemia, and regional peripheral lymphadenopathy. Euthanasia was performed at 16 weeks PI.

Post-mortem examination of the two clinically ill SHIV-33Anef-infected macaques with disease was characterized by severe widespread lymphoid depletion and multiple opportunistic infections. Both animals showed thymic atrophy and severe gastrointestinal lesions, which were characterized by marked diffuse villous blunting and atrophy, and severe, diffuse gastrointestinal cryptosporidiosis. Pulmonary *Pneumocystis carinii* infection was also present. Mmu 28872 showed milder SIV-related pathologic lesions. In addition to lesions common to both monkeys, Mmu 28872 had severe gastrointestinal infestation with *Trichomonas* sp, which accounted for a malabsorption syndrome, weight loss, and hypoproteinemia. Bone marrow hypoplasia, was observed and explained the nonregenerative anemia noted at Weeks 4, 15, and 16 PI. Histiocytic-lymphocytic SIV-related encephalitis with synctia formation was present in the brain of Mmu 28872. Histopathologic lesions of Mmu 28815 were severe and were dominated by widespread gastrointestinal infection with *Cryptosporidia* sp. These organisms extended into the pancreas and liver, causing pancreatitis, liver abscessation, and peritonitis; and were also found in the lung associated with bronchopneumonia. Disseminated widespread lymphocytic-histiocytic infiltrates were present in the skin, salivary glands, gastrointestinal tract, liver, and other soft tissues; this infiltrative lesion is typical of SIV pathogenesis. Acid fast positive *Mycobacterium* sp. organisms were also found in macrophages of the lung, cecum, and colon.

### Sequence changes in HIV-1 *nef* during SHIVnef infection in macaques

Because Mmu 27747 showed increased viral load at 24 weeks PI with SHIV-33nef and progressed to SAIDS, we hypothesized that mutations important for pathogenesis developed *in vivo* in the HIV-1$_{SF}$33 *nef* gene. To examine evolution of *nef in vivo, nef* sequences were analyzed at the early stage of infection (8 weeks PI) and at two time points during the chronic stage (32 and 53 weeks PI). Importantly, sequence comparisons were made in *nef* between Mmu 27747, the animal exhibiting SAIDS, and Mmu 27621, the animal with low virus load and no disease. In addition, the HIV-1$_{SF}$2 *nef* gene was examined in the asymptomatic SHIV-2nef-infected macaques to determine whether alterations, such as deletions, in this *nef* allele could explain the lack of pathogenicity.

The pattern of amino acid sequence changes in the animal, Mmu 27747, with high virus load after infection with SHIV-33nef, was complex. At 8 weeks PI, only 2 of 10

clones exhibited no changes from prototype input *nef.* The remaining 8 clones had one to three amino acid changes throughout *nef;* two clones acquired a premature stop codon at either position 84 or 140 (Fig. 5). By 32 weeks PI, when virus load approached high levels, 8 *nef* clones each contained from three to nine changes (Fig. 5). Consistent changes were as follows: val to ile at position 15 (7 clones), his to tyr at position 39 (6 clones), ala to thr at position 48 (4 clones), ala to asp at position 52 (5 clones), leu to val at position 75 (7 clones), and ile to val at position 113 (8 clones). At 53 weeks PI, 14 *nef* clones were analyzed, and identical changes to those detected at 32 weeks were noted at positions 15, 39, 75, and 113 (Fig. 5). Positions 48, 49, and 52 were altered in most of the *nef* clones at 53 weeks PI; however, these alterations involved substitution of two or more different amino acids at each of these three positions. An important issue is whether these sequence changes altered Nef function(s). In *in vitro* kinase assays, Nef of SHIV-33A associated with NAK and PAK to similar levels as did Nef of SHIV-33 (Fig. 2B). Thus passage of the chimeric virus through Mmu 27747 did not affect levels of Nef protein or the ability of Nef to associate with NAK and PAK in the kinase assays.

In contrast, the *nef* gene in the SHIV-33nef-infected animal, Mmu 27621, showing low virus load and no clinical signs throughout the course of infection, contained very few changes in amino acid sequence at 32 weeks PI. Four clones, obtained by PCR amplification, each displayed only one amino acid change compared to the prototype HIV-1$_{SF}$33 *nef* sequence (Fig. 6). In the 53-week samples from this animal, each of eight *nef* clones displayed one to six amino acid differences from the prototype. None of the Nef sequence changes in virus from Mmu 27621 matched any of the changes in virus from Mmu 27747 (Figs. 5 and 6).

To provide an additional comparison for evaluating the sequence changes in Nef of SHIV-33nef *in vivo,* virus was recovered from Mmu 26120 and Mmu 26167 at 32 weeks PI with SHIV-2nef, and the *nef* genes were cloned and sequenced. Mmu 26167 *nef* clone 2.32-41 contained 4 amino acid changes: trp to cys at position 13, glu to gly at position 112, glue to lys at position 158, glue to lys at position 159. Mmu 26120 two *nef* clones, 2.32-4 and 2.32-29, each contained a change of trp to tyr at position 106. Importantly, these changes in Nef of virus recovered from the SHIV-2nef-infected animals did not match any changes in the SHIV-33nef- or SHIV-33Anef-infected animals (Figs. 5 and 6).

To determine whether there was continued selection *in vivo* for sequence changes in *nef* of SHIV-33Anef, *nef* sequences were analyzed in the three animals with SAIDS, Mmu 28815, Mmu 28872, and Mmu 28883. Figure 7 shows that all clones from these three animals retained the alterations, his to tyr at position 39, and the ile to val at position 113; these changes were present in all clones of SHIV-33Anef recovered from necropsy of Mmu 27747. Additional consistent changes in the *nef* genes of these serial passage recipients were val to ile at position 15, and ala to either pro or asp at position 52 (Fig. 7). The change of leu to val at position 75 was retained in *nef* of Mmu 28815 but not in *nef* of Mmu 28883 at 32 weeks after infection. These SHIV-33Anef-infected animals also harbored a predominance of *nef* clones with alteration of lys to arg at position 70.

## DISCUSSION

This study examined SHIVnef chimeras in juvenile rhesus macaques with the aim of developing an animal model to analyze HIV-1 Nef function *in vivo.* A summary of the animal inoculations and clinical outcomes is diagrammed in Fig. 8. One of two macaques inoculated with SHIV-33nef showed high virus load at 24 weeks PI and progressed to fatal SAIDS at 53 weeks PI. Our *in vivo* double-labelling studies showed that macrophages were the predominant cell expressing viral RNA in lymphoid tissues of SHIV-33nef-infected Mmu 27747 (Fig. 4). Only low numbers of virally infected T cells were detected. This finding is supported by the recent cell culture studies, which demonstrated that Nef induces macrophages to produce CC-chemokines that mediate lymphocyte chemotaxis and activation, thus enhancing the efficiency of viral infection of T cells and presumably setting the stage for T cell depletion (Swingler *et al.,* 1999).

Both macaques infected with SHIV-2nef exhibited low virus load and remained healthy for >83 weeks PI. Thus the *nef* allele of HIV-1$_{SF}$2 may be less efficient than the HIV-1$_{SF}$33 *nef* allele at enabling the chimeric virus to establish a high viral replication rate to generate a pathogenic variant. Interestingly, HIV-1$_{SF}$2 was isolated from a patient in the asymptomatic stage of infection, whereas HIV-1$_{SF}$33 was recovered from a patient in the terminal stage of AIDS. Both SHIV-2nef and SHIV-33nef replicated to levels similar to SIVmac239nef+ in cultures of activated rhesus macaque PBMC, and both chimeras produced Nef proteins that associated with NAK and PAK to similar levels. In a large proportion of adult macaques infected with a clone of SIVmac239 bearing a large deletion in *nef,* additional deletions accumulated in the remaining *nef* sequences over time *in vivo* (Kirchhoff *et al.,* 1994). The *nef* region of SHIV-2nef in viruses recovered at 32 and 53 weeks PI of both Mmu 26120 and Mmu 26167 was analyzed by PCR amplification and DNA sequencing; these studies revealed that the *nef* gene was intact in virus in both of these animals. Accordingly, this finding suggests that HIV-1$_{SF}$2 *nef* expressed some level of Nef function *in vivo* because the *nef* gene of the chimeric virus, SHIV-2nef, did not show evidence of extensive deletion (Kirchhoff *et al.,* 1995).

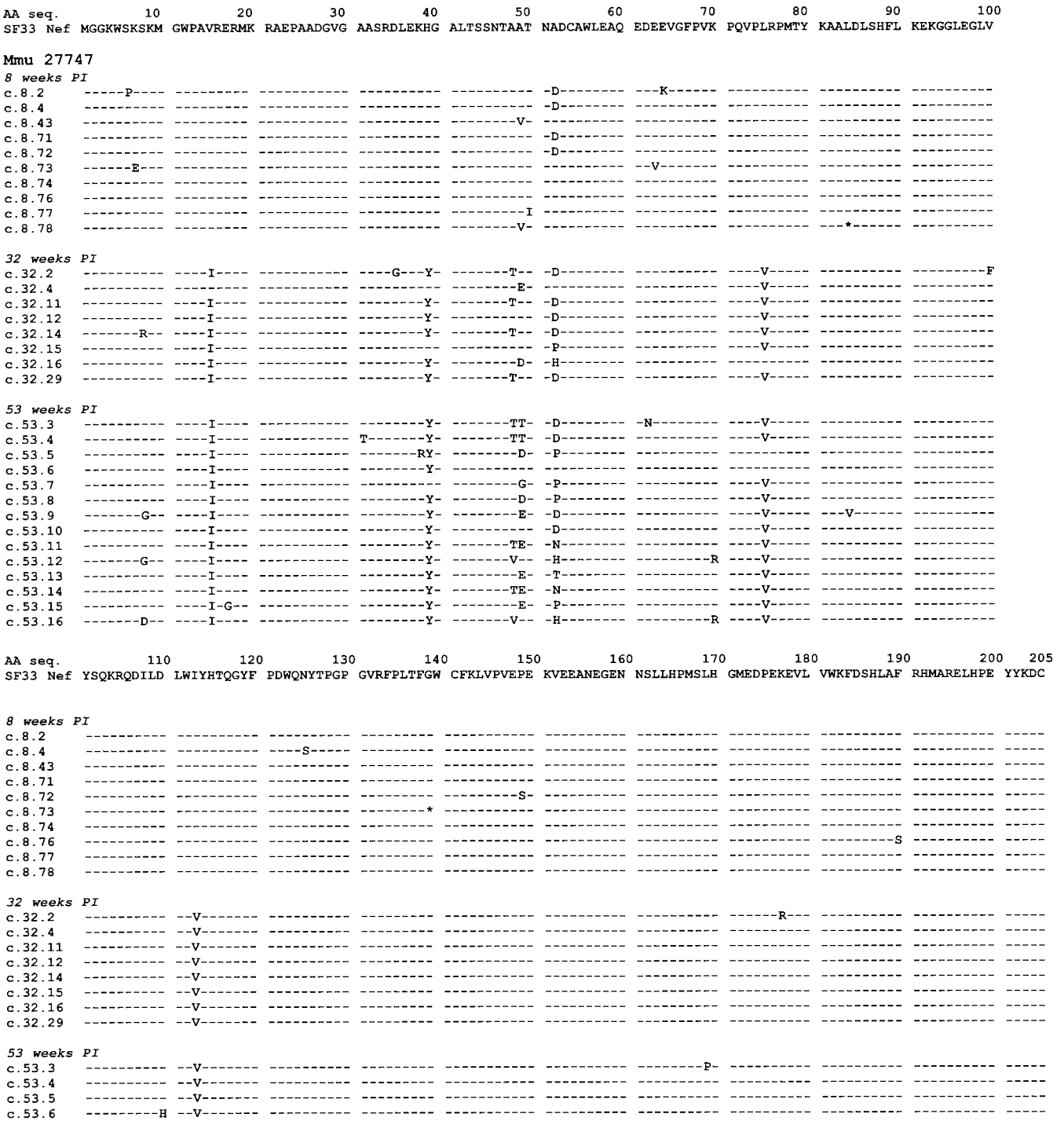


FIG. 5. Alignment of Nef sequences of virus recovered from Mmu 27747, which was infected with SHIV-33nef and developed SAIDS. The amino acid sequence for HIV-$1_{SF33}$ Nef is shown above the solid line. Amino acids are designated by single letter code. Dashes indicate amino acid identity with HIV-1SF33. *, premature stop codons. Sequences for individual PCR clones are designated by the week PI and clone number.

```
AA seq.          10         20         30         40         50         60         70         80         90        100
SF33 Nef   MGGKWSKSKM GWPAVRERMK RAEPAADGVG AASRDLEKHG ALTSSNTAAT NADCAWLEAQ EDEEVGFPVK PQVPLRPMTY KAALDLSHFL KEKGGLEGLV

Mmu 27621
32 weeks PI
c.32a.10   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.32a.17   -------G-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.32a.26   -------G-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.32a.49   -------G-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

53 weeks PI
c.53a.14   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.53a.211  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.53a.212  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c.53a.215  ---------- ---------- ---------- -------R-- ---------- ---------- ---------- ---------- ---------- ----------
c.53a.216  ---------- ---------- ---------- -------R-- ---------- ---------- ---------- ---------- ---------- ----------
c.53a.218  ---------- -G-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c53a.2110  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
c53a.2112  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------


AA seq.         110        120        130        140        150        160        170        180        190        200   205
SF33 Nef   YSQKRQDILD LWIYHTQGYF PDWQNYTPGP GVRFPLTFGW CFKLVPVEPE KVEEANEGEN NSLLHPMSLH GMEDPEKEVL VWKFDSHLAF RHMARELHPE YYKDC

Mmu 27621
32 weeks PI
c.32a.10   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
c.32a.17   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
c.32a.26   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
c.32a.49   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

53 weeks PI
c.53a.14   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c.53a.211  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
c.53a.212  ---------- ---------- ----S----- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c.53a.215  ---------- ----R----- ----S----- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c.53a.216  ---------- ---------- ----S----- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c.53a.218  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c53a.2110  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --R------- ---------- -----
c53a.2112  ---------- ---------- ----S----- ---------* ---------- ---------- ---------- ---------- --R------- --------Q-- -----

* = STOP CODON
```

FIG. 6. Alignment of Nef sequences of virus recovered from Mmu 27621, which was infected with SHIV-33nef and remained healthy. See legend for Fig. 5.

## Selection for sequence changes in HIV-1 *nef in vivo*

In previous studies, sequence changes in HIV-1 *env* genes were identified in rhesus macaques infected with SHIVenv chimeras (i.e., recombinant clones constructed by replacing the SIVmac239 *env* gene with the counterpart region of various HIV-1 clones). These sequence changes were associated with development of high viral load and progression to SAIDS (Stephens *et al.,* 1997; Karlsson *et al.,* 1998; Luciw *et al.,* 1999). Accordingly, in the present *in vivo* study, we examined *nef* gene sequences in SHIVnef-infected macaques. At 32 and 53 weeks PI, *nef* clones from Mmu 27747, the SHIV-33nef-infected animal exhibiting high virus load and SAIDS, contained three to nine amino acid changes from the prototype sequence (Fig. 5). These changes fell into three categories: (i) four amino acids, at positions 15, 39, 75, and 113, were altered, each to the same new amino acid in the majority of *nef* clones, (ii) amino acids at positions 48, 49, and 52 were altered to two or more amino acids at each position in the majority of *nef* clones, and (iii) two to four additional amino acid changes occurred throughout the *nef* sequence in each clone (Fig. 5). SHIV-33Anef, the virus recovered at necropsy at 53 weeks PI from Mmu 27747, was inoculated into four juvenile macaques (Fig. 8). All four recipient animals showed high virus loads; three have progressed to fatal SAIDS. The *nef* clones in these recipients contained the consistent changes at positions 15, 39, 75, and 113 of Nef that were observed in SHIV-33Anef recovered from Mmu 27747 (Figs. 5 and 7).

These consistent sequence changes in Nef of the chimeric viruses may be a consequence of selection for HIV-1 Nef proteins that interact efficiently with a cellular protein(s) mediating the function of this viral protein in the simian host. The mutation of his to tyr at position 39 generated a Y-X-X-L motif, which could represent a src-homology region 2 (SH2) motif (Mayer and Gupta, 1998) and/or an endocytic signal motif (Kirchausen *et al.,* 1997). The YE and PBJ alleles of SIV *nef* contain an immunoreceptor activation motif (ITAM) near the N terminus (Luo and Peterlin, 1997; Whetter *et al.,* 1998); these alleles confer rapid disease potential on SIV (Du *et al.,* 1995; Saucier *et al.,* 1998). Because most ITAMs have at least two Y-X-X-L motifs, further analysis will be required to determine whether this motif in SHIV-33Anef functions as an ITAM. Another notable change was leu to val at position 75 (Figs. 5 and 7); this leu in the prototype sequence is part of the highly conserved SH3-ligand domain (i.e., pro-x-x-pro motif) in Nef of primate lentiviruses (Saksela, 1997). It is possible that such an alteration at position 75 influences

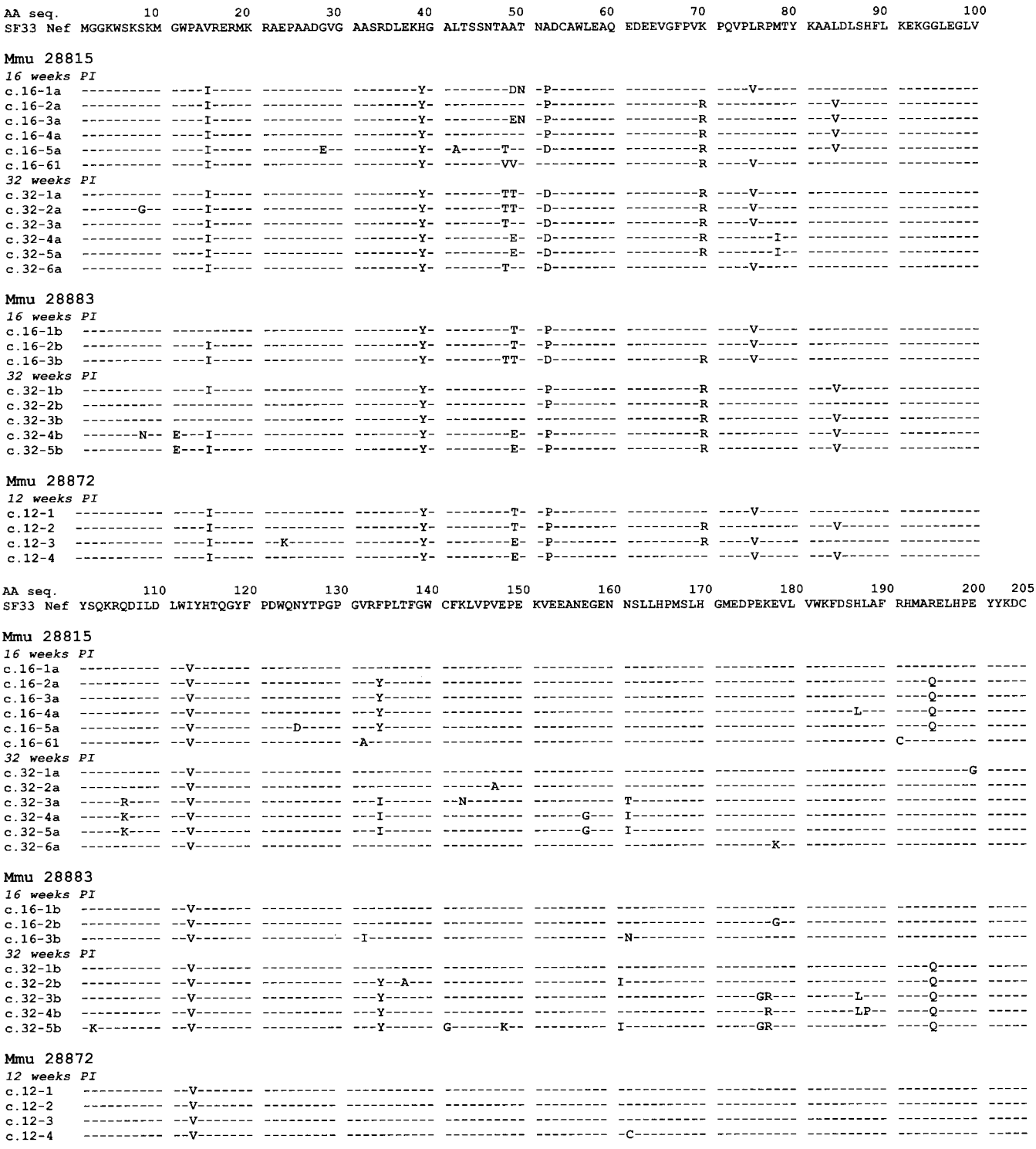


FIG. 7. Alignment of Nef sequences of virus recovered from macaques, which were infected with the recovered virus, SHIV-33Anef, and exhibited SAIDS. See legend for Fig. 5.

the affinity and/or specificity of HIV-1 Nef for a cellular signaling protein containing an SH3 domain. Positions 48, 49, and 52 were substituted with two or more different amino acids at each of these three positions; it appears that the ala residues at positions 49 and 52 were generally altered to amino acids with large side-groups (Figs. 5 and 7). The changes at these three positions are difficult to interpret because the structural model for Nef does not include the first 60 amino acids at the N terminus; this region is believed to adopt a relatively unstructured conformation under physiological conditions (Grzesiek *et al.,* 1996; Lee *et al.,* 1996).

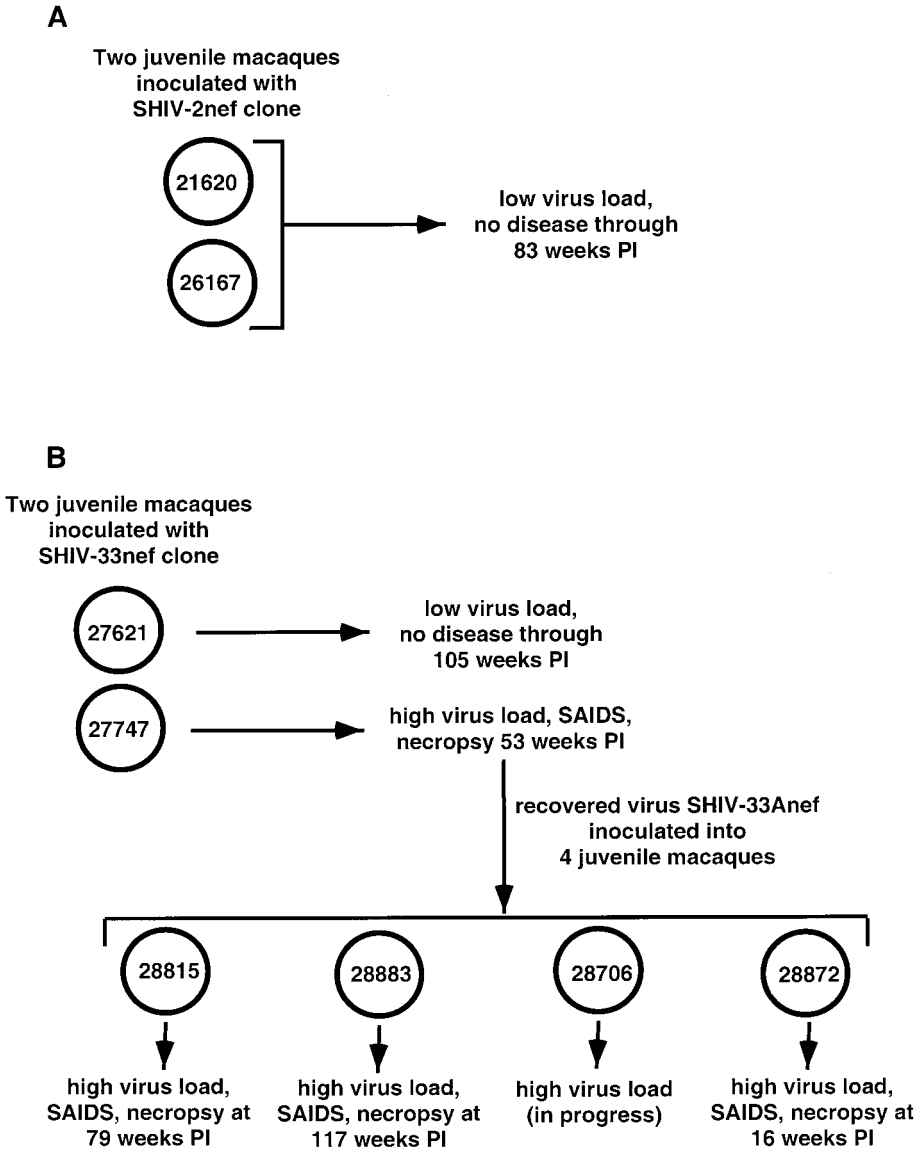


FIG. 8. Summary of macaque inoculations with SHIVnef chimeras. This figure summarizes the experimental plan and clinical outcomes of juvenile rhesus macaques inoculated with the SHIV-2nef and SHIV-33nef clones, and uncloned SHIV-33Anef. Mmu 28706, infected with SHIV-33Anef, is exhibiting high virus load and will be monitored for development of SAIDS.

It is also possible that sequence changes in Nef of the chimeric virus may be due to selection for more efficient interaction of Nef with another SIV protein. Recent findings with SHIVenv chimeras implicated a potential functional relationship between the *env* glycoprotein and Nef. Interestingly, either the YE or PBJ allele of SIV Nef was not sufficient to elicit pathogenicity in SHIVenv chimeras containing the *env* genes of HIV-1$_{HXB2}$ (Stephens *et al.,* 1997), HIV-1$_{SF162}$ (Luciw and Cheng-Mayer, unpublished results), and HIV-1$_{DH12}$ (Shibata *et al.,* 1997). These findings suggest a functional relationship between Nef and the *env* glycoprotein. Because both the *env* glycoprotein (Hunter, 1997) and Nef (Aiken, 1997; Luo *et al.,* 1998) influence virion entry into cells, a plausible scenario is that a change in one of these viral proteins may require a "compensating" change in the other to maintain full virion infectivity. Accordingly, studies on SHIVnef chimeras *in vivo* may provide novel insight on the functional interplay of viral genes. In addition, host immune responses to immunological epitopes in Nef may contribute to sequence variation *in vivo* (McMichael and Philips, 1997). HIV-1 Nef contains target epitopes for both helper and cytotoxic T-lymphocytes (CTL) (Korber *et al.,* 1995). Amino acid sequences recognized by human CTL are located throughout Nef, although the majority of CTL epitopes are located in the central region of this viral protein. It is not practical to compare Nef epitopes in a direct fashion between HIV-1-infected humans and SHIVnef-infected macaques without first establishing recognition patterns for HIV-1 Nef epitopes by macaque

CTL. Thus the SHIVnef system offers opportunities to investigate the role of immune selection on HIV-1 Nef and thereby to better understand immunological factors in viral adaptation and persistence.

### Utility of SHIVnef for *in vivo* analysis of Nef function

Whether amino acid changes in HIV-1 *nef* acquired during *in vivo* passage of SHIV-33nef are both necessary and sufficient for pathogenesis remains to be determined. Nucleotide sequence changes were not detected in the polypurine tract (the primer for initiation of viral plus-strand DNA during reverse transcription) or 5′ end of the U3 region (containing sequences recognized by the viral integrase) of SHIV-33Anef (these authors, data not shown). It is possible that a change(s) in SIVmac sequences of the chimeric virus, in addition to changes in HIV-1SF33 *nef,* may also be important for adaptation of SHIV-33Anef to the macaque host. Novel chimeric clones, containing HIV-1 *nef* alleles from SHIV-33Anef, can be constructed and analyzed in macaques to determine the significance of the changes, observed in HIV-1SF33 *nef,* for high virus load and pathogenesis. Additionally, a measurable phenotype(s) based on an *in vitro* assay for Nef function (i.e., in tissue culture cells) will be important for elucidating molecular mechanisms of SHIVnef immunodeficiency.

During the course of the studies reported in our paper, another group reported the *in vivo* analysis of SHIVnef chimeras built from the *nef* genes of the cell-line adapted HIV-1NL4-3 strain (Alexander *et al.,* 1999). Persisting high virus load and SAIDS were observed in about half of a large group of rhesus macaques infected with SHIVnef chimeras containing the HIV-1NL4-3 nef gene. This contrasts with our finding that SHIV-33Anef persisted at high levels in four of four macaques; during the observation period of ~2 years, three of these four animals developed fatal SAIDS. It is possible that the nature of the HIV-1 *nef* allele (NL4-3 *vs* SF33) influences virus load and disease progression.

Evolution of HIV-1 in humans and SIV in susceptible macaques involves changes in viral sequences (Wolinsky *et al.,* 1996 and references therein); and these changes, largely affecting *env* gene functions, appear to correlate with progression to AIDS (Connor and Ho, 1994; Rudensey *et al.,* 1998). Several studies reported changes in *nef* sequence in HIV-1-infected individuals and chimpanzees in relation to disease progression (Huang *et al.,* 1995; Mwaengo and Novembre, 1998; Salvi *et al.,* 1998) and/or organ tropism (McPhee *et al.,* 1998); however, no firm conclusions have been made on the importance of such changes in *nef* to the development of AIDS. Accordingly, the results in this study, on the nonpathogenic and pathogenic pair SHIV-33nef and SHIV-33Anef, respectively, set the stage for analyzing functions of HIV-1 Nef and its domains in viral persistence and pathogenesis in non-human primate models for AIDS. Additionally, SHIVnef chimeras can now be used to evaluate drugs and therapies targeted to HIV-1 Nef in the non-human primate model for AIDS.

## MATERIALS AND METHODS

### Cell and virus culture

Peripheral blood mononuclear cells (PBMC) were obtained from healthy rhesus macaques free of simian type-D retroviruses (SRV), SIV, and simian T-lymphotropic virus (STLV). These cells, purified from whole blood by Ficoll–Hypaque centrifugation, were stimulated with staphylococcal enterotoxin A (SEA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS) and 10% interleukin-2 (Collaborative Research, Bedford, MA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). CEMx174 cells, a human hybrid T-B cell line permissive for primate lentiviruses (provided by Dr. J. Hoxie, University of Pennsylvania, Philadelphia, PA), were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics. Titered stocks of cell-free viruses were propagated in a 50 ml culture of CEMx174 cells. Culture supernatant was collected at 5–6 days after infection (when ~25% of cells displayed cytopathic effects), passed through a 0.22 μm filter, and frozen in 1 ml aliquots. The 50% tissue culture infectious dose ($TCID_{50}$) of these viral stocks in CEMx174 cells was determined by procedures described previously (Marthas *et al.,* 1993).

### Construction of chimeric SHIV-2nef and SHIV-33nef

The cloned genome of SIVmac239 was modified to accommodate substitution of the SIV *nef* gene with a DNA fragment containing HIV-1 *nef.* The *nef* genes of the T lymphotropic, cytopathic HIV-1SF2, and HIV-1SF33 isolates were selected; the former virus was recovered from an asymptomatic HIV-1-positive individual prior to the onset of AIDS, and the latter was isolated from an individual with AIDS. These two *nef* genes differ by 24 of 206 amino acids, primarily in the amino terminus. Because the start of the SIVmac239 *nef* gene overlaps coding sequences near the 3′ end of the *env* gene, the SIVmac239 molecular clone (GenBank Accession No. M33262) was modified by mutating two ATG codons (positions 9334 and 9352) near the 5′ end of *nef* (Fig. 9). These changes were necessary to preclude synthesis of hybrid (SIV/HIV-1) Nef proteins. The 3′ portion of the SIVmac239 genome, from the *Sph*I site in *vpr* through the 3′ long terminal repeat (LTR) was cloned into pGEM-7Z to produce pVP-2. Oligomutagenesis was performed to mutate the T in the two ATG codons at the beginning of SIV *nef* to C; this was done with the Muta-Gene Phagemid Kit from BioRad (Richmond, CA) (Ausubel *et al.,*

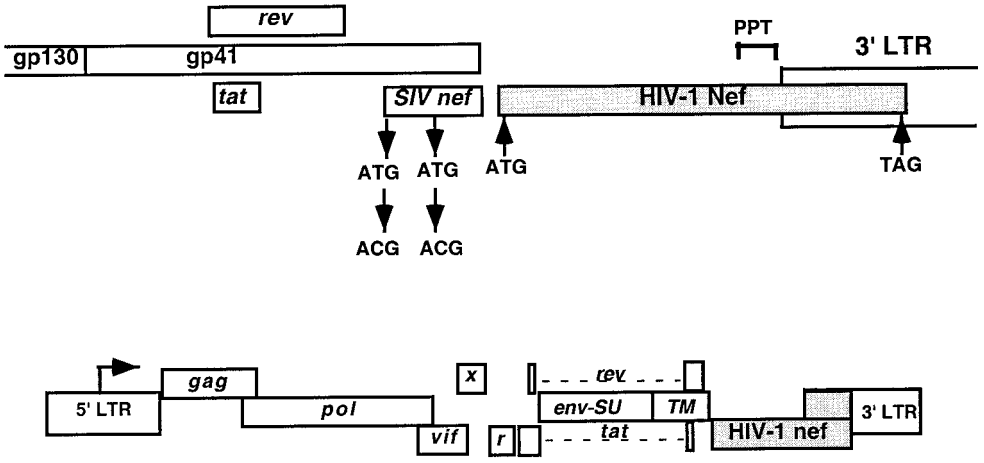


FIG. 9. Construction of the SHIVnef chimeric viruses. The 3′ end of the SHIVnef constructs, including the 3′ end of SIVmac239 *env* through the 3′ LTR, is shown in the top panel. In SIVmac239, the *nef* gene overlaps with the 3′ portion of the *env* gene. The two SIV *nef* ATG start codons (positions 9334 and 9352) were mutated by oligomutagenesis to ACG to preclude translational initiation in SIV *nef* sequences. The region between the end of *env* (position 9499) and extending through the 3′ LTR (position 10,083) was replaced with the *nef* gene of either HIV-1SF2 or HIV-1SF33. Accordingly, the 3′LTR is a hybrid of HIV-1 and SIV sequences. The bottom panel shows the full proviral form of the SHIVnef clone.

1993). These mutations were verified by DNA sequencing. Additionally, a double-stranded oligonucleotide polylinker was installed in the modified pVP-2 clone, between the end of the env gene (position 9499) and the *Stu*I site (position 10,085) in the U3 portion of the LTR. This polylinker contains *Sal*I, *Afl*3, *Hin*fI, *Nde*I, and *Stu*I sites and the resulting plasmid was designated pVP-2B. DNA fragments with the HIV-1SF2 (GenBank Accession No. K02007) and HIV-1SF33 (GenBank Accession No. M38427) *nef* genes were obtained by PCR amplification from the parental HIV-1 clones. For HIV-1SF2, the following oligonucleotide primers were used: for the 5′ end of *nef* 5′A**GTCGAC**GAATTAGACAGGGCTTGGAAAGG3′ (*Sal*I site in bold); for the 3′ end of *nef* 5′G**AGGCCT**TGTAGAAAGCTCGATGTCAGC3′ (*Stu*I site in bold). For HIV-1SF33, the following oligonucleotide primers were used: for the 5′ end of *nef* 5′G**GTCGAC**TTTGCTATAAGATGGGTGGCAAGTGG3′ (*Sal*I site in bold), for the 3′ end of *nef* 5′GAGTACTAGAAAGACTGCTGACATCG**AGGCCT**AA**CTCGAG**G3′ (*Stu*I and *Xho*I sites in bold, respectively). Because the 5′ and 3′ primers contained a *Sal*I and *Stu*I site, respectively, the PCR amplified *nef* DNA fragments were cloned into the polylinker of pVP-2B by *Sal*I/*Stu*I digest to produce pVP-2nef2 and pVP-2nef33. DNA sequencing was performed on the clones to verify the sequence of the polylinker and HIV-1 *nef* genes. To produce the full-length SHIVnef recombinant viruses, the linearized 5′ half of SIVmac239, designated pVP-1, was ligated with T4 DNA ligase to linearized pVP-2nef2 or pVP2nef33 to yield SHIV-2nef or SHIV-33nef, respectively. To generate the biologically active virus, each of the full-length SHIVnef recombinants were transfected by electroporation into CEMx174 cells using a Bio-Rad Gene Pulser and Extender (Bio-Rad, Richmond, CA) as previously described (Banapour *et al.,* 1991).

### *In vitro* kinase assay

*In vitro* kinase assays were performed, as described previously, on immunoprecipitates obtained from lysates of virally infected CEMx174 cells using either rabbit anti-HIV Nef antibody or rabbit anti-rat PAK-1 antibody (Santa Cruz Technologies, Santa Cruz, CA) (Sawai *et al.,* 1996).

### Inoculation of rhesus macaques with SHIVnef recombinants

Eight healthy, colony-bred retrovirus-free juvenile rhesus macaques, *Macaca mulatta,* between the ages of 2.5 and 2.9 years and weighing 3.6–4.6 kg were used. All of the monkeys were negative for antibodies for SRV, SIV, and STLV-1. Animals were housed and cared for at the California Regional Primate Research Center (CRPRC) at Davis, CA in accordance with American Association for Accreditation of Laboratory Animal Care Standards. Macaques were anesthetized with ketamine hydrochloride to obtain blood and lymph node samples. Before inoculation, 20 ml of blood was collected by venipuncture for plasma, complete blood counts (CBC), and CD4/CD8 T-cell immunophenotyping by flow cytometry. Also, prior to virus inoculation and at serial time points after inoculation, peripheral lymph nodes were obtained by excisional biopsy and portions of lymph nodes were fixed in 10% buffered formalin, flash frozen in liquid nitrogen, and preserved in OCT. Animals were observed daily and weighed once weekly by the CRPRC veterinary staff. Complete physical examinations were performed before and after inoculation to clinical illness. When clinical signs of SAIDS were severe, animals were euthanized with an overdose of sodium pentobarbital. Titered stocks of cell-free SHIV-2nef and SHIV-33nef prepared in

CEMx174 cells were used to intravenously inoculate each macaque at 1000 TCID50 of virus.

### Measures of viral load and antiviral antibodies

For measuring plasma viremia, serial 10-fold plasma dilutions were made in tissue culture medium and dispensed into 24-well microtiter plates containing $2.5 \times 10^5$ CEMx174 cells (Marthas *et al.,* 1993). For measuring cell-associated viral loads, $10^6$ PBMC or lymph node mononuclear cells (LNMC), and serial 10-fold dilutions of these cells, from each infected macaque were cocultured with $2.5 \times 10^5$ CEMx174 cells per well with 4 wells per dilution. These cultures were observed for cytopathology by light microscopy, and culture medium was assayed for SIV $p27^{gag}$ antigen by ELISA (Coulter, Hialeah, FL) to monitor virus production. Titers were calculated by the method of Reed and Meunch to determine the number of infected PBMC per $10^6$ total PBMC (Marthas *et al.,* 1993). In addition, levels of viral RNA in plasma samples of infected macaques were determined by bDNA assay (Chiron Corp., Emeryville, CA) (Dailey *et al.,* 1995). To measure anti-SIV antibodies, serial twofold dilutions of plasma were evaluated using an ELISA containing purified whole HIV-2 that crossreacts with SIV (Genetic Systems, Seattle, WA).

### Hematologic evaluation and T-cell immunophenotyping

CBC were performed by a standard automated method (Biochem Immunosystems, Allentown, PA) on EDTA anticoagulated blood. CD4+ and CD8+ T-cell immunophenotyping was performed by flow cytometry using a two-color whole-blood lysis technique (Q-Prep, Coulter, Hialeah, FL) (Reimann *et al.,* 1994). Fifty microliters of whole blood was incubated in the dark at 25°C with anti-CD4 (Leu3a; Becton Dickinson, Mountain View, CA) and anti-CD8 (Leu2a, Becton Dickinson, Mountain View, CA) specific monoclonal antibodies according to manufacturer's instructions and were analyzed by flow cytometry using a FACScan (Becton Dickinson).

### Detection of virus in tissues

Combined *in situ* hybridization/immunohistochemistry was performed as previously described (Mandell *et al.,* 1995). A 4.5-kb SIVmac239 genomic DNA fragment containing the *gag* and *pol* regions was radioactively labeled with [$^{35}$S]CTP by random priming in a DNA polymerase reaction to synthesize a SIV DNA probe with a specific activity of $\geq 1 \times 10^8$ cpm/$\mu$g. To detect both SIV DNA and RNA, coverslipped slides were heated at 95°C for 7 min., cooled on ice for 3 min., then incubated overnight at 37°C in humidification chambers. *In situ* hybridization experiments were repeated at least three times to verify consistency of results. Control samples included 4% paraformaldehyde fixed SIV-infected and uninfected cultured CEMx174 cells, hybridization of lymph node and gastrointestinal tissue from SIV-infected and uninfected monkeys, hybridization with probe containing only the pSP64 vector, and RNase treatment of tissue before hybridization. Monocyte/macrophages and T lymphocytes were localized, respectively, using the HAM-56 monoclonal antibody specific for macrophages (DAKO Corporation, Carpentieria, CA) and a polyclonal antibody specific for CD3+ T-cells (DAKO Corporation). These antibodies have been validated for use on uninfected and infected rhesus macaque tissues (Mandell *et al.,* 1995).

### Recovery and sequence analysis of HIV-1 *nef* from infected macaques

At various times after inoculation, LNMC and PBMC were collected from macaques infected with SHIV-2nef, SHIV-33nef, or SHIV-33Anef. DNA was extracted using a commercial kit according to manufacturer's instructions (QIAmp Blood Kit, Qiagen, Chatsworth, CA). The region containing HIV-1 *nef* was amplified by nested polymerase chain reaction (PCR) using standardized protocols. Details of PCR amplification, conditions and DNA sequencing are available upon request from the authors. Importantly, multiple PCR amplifications were performed to obtain *nef* clones from the animals in this study.

## ACKNOWLEDGMENTS

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*Note added in proof.* The macaque infected with SHIV-33Anef, Mmu 28883, was euthanized at 117 weeks postinoculation because of SAIDS-related disease. This animal showed moderate to high viral load throughout infection. Prior to euthanasia, Mmu 28883 developed marked weight loss, diarrhea and colitis, and an extensive rash and dermatitis. At necropsy, widespread lymphoid hyperplasia was noted in all lymphoid organs (lymph nodes, thymus, spleen, gastrointestinal and pulmonary lymphoid tissues) as well as early lymphoid depletion in the spleen. In addition to generalized lymphoid hyperplasia, the diffuse, extensive dermatitis with secondary bacterial infection and the increased severity of the ulcerative colitis lesions are related to infection with immunodeficiency lentiviruses in primates. Virus load at necropsy of Mmu 28883, measured by bDNA assay, was in the high range at $2.2 \times 10^6$ viral RNA equivalents per ml of plasma (Fig. 3B). The remaining SHIV-33Anef-infected animal, Mmu 28706, continues to maintain high virus load, greater than $5 \times 10^5$ viral RNA equivalents per ml of plasma at 52 weeks after infection (Fig. 3B). During the preparation of this paper, another report on SHIVnef pathogenesis in macaques was published by Kirchhoff and colleagues (*J. Virol.* **73,** 8371–8383, 1999).

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